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Published Monthly except August and September at
North Queen St. and McGovern Ave., Lancaster, Pa.

Entered as second-class mail matter February 4, 1935, at the Post Office at Lancaster, Pennsylvania, under the Act of March 3, 1879.

THE BOTANICAL REVIEW

Founded in 1935 by

HENRY ALLAN GLEASON EDMUND H. FULLING

Managed, edited and published at
The New York Botanical Garden
by
EDMUND H. FULLING

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Articles are obtained primarily by invitation. Unsolicited manuscripts are also welcome. In all cases, however, the editor reserves the right to accept, reject or suggest revisions in submitted material. All manuscripts should be on double-space typewritten pages, with references and citations in accordance with the customary style of THE BOTANICAL REVIEW. Mention of authors' names in the text should be reduced to a minimum, and reference to their works made by citation numbers. Emphasis of particular authors' names, however, and the desirability of variation render occasional departure from this policy justified.

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All correspondence should be addressed to THE BOTANICAL REVIEW, at N. Queen St. and McGovern Ave., Lancaster, Pa., or Fordham P. O., New York, N. Y.

THE BOTANICAL REVIEW

VOL. XV MARCH, 1949 No. 3

GERMINATION INHIBITORS

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INTRODUCTION

Ontogeny is not a continuous process of development, but is interspersed with periods during which development is completely or partially inhibited. The egg cell and the endosperm nucleus before fertilization, the embryo in most ripe seeds, and winter and summer rest periods of trees and shrubs are good examples of such inhibition phases belonging to the normal developmental cycle. Sometimes such inhibitions are brought about by environmental conditions, more often by conditions within the plant itself.

The causes of these inhibitions are manifold. One of them—the one dealt with in this paper—lies in the production of substances inhibiting particular physiological processes which are indispensable for normal development. These inhibitors are very interesting because:

(*a*) They are equally as important in the ontogeny of plants as are growth hormones and other stimulating substances.

(*b*) When excreted they inhibit the development of other species in their surroundings; *Agrostemma Githago,* for instance, cannot germinate in fields of *Beta* because *Beta* excretes a substance which inhibits the germination of *Agrostemma* seeds. Such effects show a striking similarity with the natural function of antibiotics excreted by soil fungi or bacteria which inhibit or destroy other soil organisms in the same locality.

(*c*) They present a problem of the widest physiological significance. Apparently all physiological reactions are governed by a set of "positive" as well as by one of "negative" catalysts whose function it is to delay or inhibit reactions. This was clearly seen as far back as 1903 when Czapek (30) called these substances "negative catalysts" or "antiferments".

This paper deals only with excretory germination inhibitors. All other reasons for inhibition of germination and for primary or secondary (induced) dormancy—*e.g.*, rudimentary embryos, mechanical resistance to expansion of the embryo, seed coats impermeable to water (29, 140)—are excluded.

Germination inhibitors are substances produced by plants or substances of related structure not found in plants which inhibit or delay the germination of seeds of the same or other species. This definition is purely phenomenological, since a better definition, for lack of appropriate knowledge, and based on the inhibitory mechanism, is not possible today. One weak spot in this definition is that we are not sure, as will be seen later, whether germination itself or merely the first phase of subsequent growth is inhibited. If only growth is inhibited, the whole problem would merge into the problem of growth inhibitors. For the time being, then, we may speak of germination inhibitors as substances which from observation have been found to inhibit germination.

TEST METHODS

The prevalent method for testing germination inhibitors is to germinate various seeds in Petri dishes on filter paper moistened by the solutions to be tested (79, 80, 135, 136). Sometimes the parts containing the inhibitors are put into the same dishes with the seeds (148). For the tests different seeds have been used, *e.g.*, wheat (79, 80), *Lepidium sativum* (38, 74), *Papaver orientale* and *Avena sativa* (38). Although many seeds, *e.g.*, *Lepidium sativum*, are more sensitive to inhibitors than wheat grains, the use of wheat is to be recommended, since pure standard varieties of the same quality, which germinate 91% to 100%, are easily obtainable. Oat grains, for which the same holds true are not so well suited, since their glumes already contain an inhibitor. Experiments by the present author to establish a test by using pollen grains as test objects have failed, despite the fact that germination of them is inhibited by all germination inhibitors so far tested (82). Although they are much more sensitive to inhibitors than seeds, their use in tests is impractical because it is nearly impossible to obtain good uniform material the whole year round and it is not easy to create conditions under which their germination invariably reaches 90% to 100% in the controls.

A different test method was used by Froeschel (45–47). He put the materials to be tested for inhibitory content in front of the test seeds. The water supply was thus unilateral, having to pass the materials to be tested in order to reach the seeds. This method is excellent to demonstrate the inhibiting effect but cannot be employed when the relation between inhibitor concentration and inhibition is to be established.

When the effect of inhibition on growth is to be tested, fresh or dry weight of the seedlings or radicles (79, 80) or the length of the radicles (80) is determined in a test and control experiment.

DISTRIBUTION AND LOCALISATION

A perusal of Table 1 shows that germination inhibitors are found in the most different families of the plant kingdom. Their presence seems to be a widely spread phenomenon which is not restricted to phanerogamous plants. Spores and gemmae of cryptogams show an inhibited germination very similar to that of tomato seeds inside the fruit. The gemmae of *Marchantia* do not sprout inside their cupules, and spores of *Funaria hygrometrica* do not germinate within their sporogonia (111). Inhibitors are not confined to seeds or fruit but occur in various parts of plants according to the species:

Fruit

Pulp: pear, apple, *Poterium spinosum, etc.*

Juice: Tomato, *Lonicera, etc.*

Coat: *Triticum, Helianthus, Fagopyrum, etc.*

Seeds

Coat: cabbage, lettuce, *etc.*

Embryo: *Helianthus.* Here a strong inhibition is exerted by all parts of the embryo when they are prepared from non-soaked seeds. When taken out of soaked seeds only the cotyledon inhibits; the radicle inhibits at first but later stimulates; the plumule stimulates (123).

Endosperm: *Iris* (117).

Leaf sap: *Phragmites,* spinach, *etc.* The inhibiting effect of leaf sap was overlooked for a long time (80), although Magnus (97) long ago reported the germination inhibition exerted by the leaf sap of *Phacelia* and *Pelargonium.*

Bulbs: onion, garlic.

Roots: carrot, horseradish, radish, *etc.*

TABLE 1

LIST OF KNOWN PLANT-PRODUCED GERMINATION-INHIBITORS

Species producing inhibitor	Part of plant containing inhibitor	Authors by whom inhibition was first observed
Agrostemma Githago		13
Allium Cepa	bulb juice	Orig.
A. sativum	" "	Orig.
Ammi Visnaga	fruit	Orig.
Amygdalus communis	seeds	148
Anona sp.	leave sap	80
Apium graveolens	seeds	135
Arachis hypogea	leave sap	80
Armoracia rusticana	" "	80
	root sap	Orig.
A. sativa	fruit	123, 135
Beta saccharifera	fruit coat	47, 135, 136, 144
B. vulgaris (beet root)	leave sap	80
	fruit (all parts)	38, 45, 46, 47
B. vulgaris (mangel)	leave sap	80
B. vulgaris (red beet)	" "	80
Brassica caulocarpa	" "	80
	tuber sap	Orig.
B. nigra	seeds	Orig.
B. oleracea var. *capitata*	leave sap	80
	seed coat	27, 28
Capsicum annuum	fruit	111
	fruit juice	80
C. frutescens var. *cerasiforme*	leave sap	80
Carica Papaya	fruit juice	112
Chenopodium ambrosioides	fruit	148
Cicer arietinum	seeds	164
Citrus Aurantium (*C. sinensis*)	fruit juice	138, 139, Orig.
C. Limonia	" "	138, 139, Orig.
C. maxima	" "	Orig.
C. nobilis var. *deliciosa*	" "	138, 139
Coffea arabica	seeds	148
Coriandrum sativum	fruit	148
Cucumis Melo	fruit juice	135, 139
C. Melo var. *cantaloupensis*	" "	135
C. sativus	leave sap	80
	fruit juice	71
Cucurbita Pepo	seeds	71
	seed coat	71
	embryo	71
	fruit juice	139
Cynanchum acutum	leave sap	80
Cyperus Papyrus	" "	80
Daucus carota var. *sativa*	" "	80
	root sap	Orig.
Ecballium elaterium	fruit juice	Orig.
Ephedra campylopoda	fruit	133
Eryobotrya japonica	leave sap	80

TABLE I—(*Continued*)

LIST OF KNOWN PLANT-PRODUCED GERMINATION-INHIBITORS

Species producing inhibitor	Part of plant containing inhibitor	Authors by whom inhibition was first observed
Erucaria boveana	fruit coat	133
Fagopyrum esculentum	" "	91
Feijowa Sellowiana	leave sap	80
Ficus carica	fruit flesh	74
Foeniculum vulgare	fruit	148
Helianthus annuus	leave sap	80
	fruit coat, seed	123
Hirschfeldia incana	fruit coat	165
Hordeum vulgare	fruit	83, 135
Ipomoea Batatas	leave sap	80
Iris	endosperm	117
Juniperus communis	fruit	148
Lactuca sativa	seeds	125, 126, 135
Lagenaria vulgaris	fruit flesh	110, 111
Lippia nodiflora	leave sap	80
Lonicera tatarica	fruit juice	111
Lonicera xylosteum	fruit juice	111
Lupinus mutabilis	seeds	148
Lycopus europaeus	leave sap	80
Maclura aurantiaca	fruit	111
Mangifera indica	leave sap	80
Matthiola bicornis	fruit coat	133
Nicotiana rustica	leaves	111
	fruit coat	111
Onobrychis cristagalli	pericarp	165
Papaver somniferum	fruit	111
Pelargonium	leaves	97
Persea americana	leave sap	80
Petroselinum hortense (*sativum*)	" "	80
Phacelia tanacetifolia	seeds	97, 114
	leaves	97
Phaseolus vulgaris	leave sap	80
Phragmites communis	" "	80
Phytolacca dioica	fruit	111
Pimpinella anisum	"	148
Piper nigrum	"	148
Pirus communis	fruit flesh	74
Pirus malus	" "	74
	exhalation	75
	endosperm	151
Pisum	seeds	73
Polygonum lapathifolium	leave sap	80
Poterium spinosum	fruit	149
Prunus armeniaca	fruit juice	127
Psidium guajava	leave sap	80
Punica granatum	" "	80
Raphanus sativus	root sap	Orig.
Rumex acetosa	" "	80
Silene Coeli-rosa	seeds (?)	13

TABLE I—(*Continued*)

LIST OF KNOWN PLANT-PRODUCED GERMINATION-INHIBITORS

Species producing inhibitor	Part of plant containing inhibitor	Authors by whom inhibition was first observed
Sinapis alba	fruit coat	133, 165
	pedicels	133
	seeds	Orig.
Sinapis arvensis	fruit coat	148
Solanum coagulans	fruit juice	Orig.
S. lycopersicum	" "	74, 75, 78, 79, 110, 111, 112, 118
	seeds	118
S. Melongena	leave sap	80
Sorbus aucuparia	fruit juice	82
Spinacia oleracea	leave sap	80
	seeds	135
Theobroma Cacao	"	148
Trigonella foenum graecum	"	148
Trifolium pratense	"	45, 46
Triticum sativum	fruit	107
Typha angustata	leave sap	80
Vaccaria pyramidata	seeds	13, 14
Vicia	"	73
Viola tricolor	"	148
Viscum album	leaves	52, 161, 162
	fruit	
Vithania somnifera	fruit juice	Orig.
Vitis vinifera	" "	Orig.
Zea Mays	leave sap	80

SPECIFICITY

Germination inhibitors are non-specific. The inhibitor in seeds of *Trifolium pratense* and fruits of *Beta vulgaris* inhibited the germination of seeds in 28 species belonging to 14 families (45–47), and tomato juice is effective on tomato seeds, wheat, barley, oat and maize grains (78, 79), and on seeds of *Lepidium, Sinapis, Trifolium,* (Orig.), carrot, lettuce, cabbage, sunflower (112), *etc.* The sensitivity of different seeds to the same concentration of inhibitors varies greatly, however. Tomato juice in a dilution of 1 : 25 wholly inhibits the germination of *Lepidium* seeds, whereas wheat grains start to germinate in a dilution of 1 : 4 (Orig.). One-to-four concentration of *Beta* fruit extracts inhibits the germination of seeds of *Amaranthus caudatus* completely, depresses the germi-

nation index[1] of *Lepidium* seeds to 4%, but does not affect the germination of *Ipomoea purpurea* (45, 46).

The same seeds react differently to inhibitors of different sources. The fruit juice of *Solanum coagulans,* for instance, inhibits the germination of wheat up to a dilution of 1 : 64, and the last traces of the inhibiting influence disappear only in a dilution of 1 : 500 (Orig.). The corresponding data for tomato juice are: 1 : 4 and 1 : 10, respectively (Orig.); for leaf sap of *Phragmites communis,* 1 : 2 to 1 : 10 and 1 : 50 (76); for garlic juice, 1 : 16 and 1 : 100.

INHIBITION AND OSMOTIC PRESSURE

Oppenheimer (110, 111) has discussed whether osmotic pressure of tomato juice is responsible for its inhibitory action. He came to a negative conclusion, but other authors (38, 89, 93, 99) explain the inhibition solely by the osmotic pressure of the solutions or extracts used. It is certain that in many cases osmotic pressure is one of the inhibiting factors, sometimes the most important, but there are others also.

In grape juice, where the very high (35–37 atm.) osmotic pressure is the main inhibiting agent, there is one of those other factors. In one of our experiments we measured an osmotic pressure of 35.5 atm. caused by a glucose content of 19.4%. This juice completely inhibited down to a dilution of 1 : 8 (4.4 atm.). In a dilution of 1 : 16 (2.1 atm.) the g.i. was 47%. An osmotic pressure of 4.4 atm. has no inhibitory effect or a very slight one, and an osmotic pressure of 2.1 atm. is wholly ineffective on wheat grains. When an artificial grape juice was prepared isotonic to the natural one (19.4% glucose solution), a g.i. of 5.3% was found for the 1 : 4 dilution, of 68% for the 1 : 8 dilution, and of 100.5% for the 1 : 16 dilution (Orig.). All this goes to show that aside from high osmotic pressure there is an additional inhibiting factor present.

There is also other proof to the same end. By yeast fermentation and subsequent removal of the alcohol produced, the sugar content of the juice is reduced to nil (osmotic pressure less than 0.5 atm.). This juice still inhibits completely (1 : 2 dilution gives g.i. of 17%). An isotonic glucose solution treated the same way

[1] Germination index, g.i., expressed as a percentage, is the ratio of the number of seeds germinating under the influence of an inhibitor to the number out of an equal quantity germinating in a pure water control.

shows a g.i. of 60% (1:2 dilution gives a g.i. of 89%), proving that the yeast itself does not produce an inhibitor in sufficient quantity to explain the complete inhibition of the juice (Orig.).

In sugar-beet seed extracts, where Duym and co-workers (38) state that the osmotic effect, caused by the inorganic salt constituents of the extract, is the most important factor in causing inhibition, this is true only for "decolorized" extract. In untreated extract another volatile inhibiting factor is present.

For tomato juice and other cases the facts showing that osmotic pressure is only slightly involved or of no importance whatever may be summed up as follows:

(*a*) When grains are germinated in different sugar and salt solutions (KNO_3, KCl, NaCl) isotonic to tomato juice, a slight delaying action on germination is observed, but never inhibition to the extent of that brought about by the tomato juice itself. In one case the g.i. in undiluted tomato juice was zero on the fourth day but 92% to 98% in different isotonic sugar and salt solutions (79).

(*b*) Even at a dilution of 1:5, in which the osmotic pressure of tomato juice is 1 to 2 atm., inhibition is sometimes complete; isotonic salt or sugar solutions have no effect whatever on germination (79). Kökemann (74) found the same for other fruit juices which inhibited even in 1:10 dilution where the osmotic pressure was negligible.

(*c*) By yeast fermentation the sugar content of the juice (3.6% glucose) was reduced to nil (osmotic pressure less than 0.5 atm.). This juice still inhibited germination, but in an isotonic glucose solution treated similarly no inhibition was observed (Orig.).

(*d*) There is no correlation between the height of the leaf sap osmotic pressure and its inhibitory effect. Saps of different parts of *Typha* leaves show the same inhibition, but their osmotic value varies from 9.3 to 14 atm. (80).

(*e*) In some cases the inhibitor is volatile, as in *Vaccaria pyramidata* (14), *Cucumis sativus* (71), onion and garlic (Orig.), *Brassica nigra* (Orig.).

INHIBITION AND pH

Many authors (78–80, 135, 138, 139) raise the question as to whether the acidity of fruit juices is the cause of their inhibiting action. In a very extreme case, that of lemon juice, the extremely

acid pH (about 2.5) makes it *a priori* probable that this factor may be responsible for the germination inhibition. Lemon juice with pH 2.4 inhibited the germination of wheat grains completely down to a dilution of 1:4; in a dilution of 1:8 the g.i. was 7%; in a dilution of 1:16, 32%; in a dilution of 1:32, 85%. After neutralization the respective germination figures were: dilution of 1:4, 1.5%, 1:8, 22%. This experiment shows that the pH alone was not enough to explain the inhibition, for then the influence of the neutralization would have been much more pronounced. The same is proved by germination experiments in buffer solution of different pH (78). In pH 2.27 the g.i. was 8.2%.

In experiments with artificial lemon juice (solution of 5.41% citric acid and 0.32% malic acid (pH 2.2)), complete inhibition was observed down to 1:4; and in 1:8, 6.3% germinated (131). These figures compare well with the germination percentage in natural juice. After neutralization complete inhibition was noted down to 1:4, and more or less normal germination in still lower dilutions. All this proves that pH contributes to inhibition but is not its only cause. Besides pH the osmotic pressure (23.44 atm. for natural, 34.5 atm. for neutralized undiluted juice) plays its part also, and the inhibition of the original concentration of undiluted natural juice can be explained by the combined effect of pH and osmotic pressure. But there is another factor involved whose presence is felt only where dilutions are used, since the inhibition caused by 1:8 diluted neutralized juice (4.2 atm.) cannot be explained by either pH or osmotic pressure alone.

A similar conclusion must be drawn for apple juice, when wheat grains germinated to 37% in the natural and to 45% in the neutralized juice as compared with 90% in the controls (138, 139), thus showing slight influence of the acidity but definitely ruling out the pH as the sole factor responsible for the inhibition.

For tomato juice and leaf saps the reasons why pH is of minor importance or no importance at all in causing inhibition are summed up as follows:

(*a*) Seeds germinated in buffer solution of a pH identical with that of the juices are less inhibited or not at all (66, 75).

(*b*) The inhibition is not affected or only slightly so by neutralization of the juice (79).

(*c*) There is no relation between the inhibitory action of leaf sap and its acidity. The sap with the lowest pH inhibited germination least. Saps with neutral or slightly basic pH are no less inhibitory than the acid ones (80).

CHEMICAL NATURE OF INHIBITORS

Hydrogen Cyanide ($H–C\equiv N \rightleftarrows H–N\equiv C$)

Amygdalin and other cyanophoric glucosides occur in many seeds, especially in those of the Prunaceae and Pomaceae (up to 2½%). It is hydrolyzed by emulsin to benzaldehyde, hydrogen cyanide and β-glucose. Leibach and Keil (85) were the first to point out that this process may be important in naturally occurring germination inhibitions.

Pure amygdalin under sterile conditions has a weak inhibitory effect (128). It has long been known, however, that HCN, set free from amygdalin, and the free CN group in general have a strong inhibitory effect on growth and germination (128): 0.0015% by volume suffices to inhibit the growth of *Pisum* seedlings (70), 0.1% to inhibit the germination of tomato seeds (72), 0.05% to check the growth of tomato seedlings (72), 0.024% to delay germination of *Lepidium* seeds (160). The germination of seeds is very much delayed (8, 72, 85) or completely inhibited as long as the seeds are kept in air containing HCN (narcotizing action according to 72, 85), but if removed from the HCN they retain their germinating capacity even after exposure of six days. In the presence of active charcoal the HCN set free by emulsin from the natural amygdalin is ineffective, for it is absorbed into the charcoal. The tissues of growing seedlings are much more sensitive to HCN than the seeds; the same concentration which only delays germination kills the root tissues of *Helianthus* seedlings (85).

It may therefore be assumed (72) that hydrogen cyanide is the germination inhibitor wherever seeds contain HCN or potential HCN-producing compounds. This has been proved for almond seeds. Two bitter almonds containing 1% to 3% amygdalin, placed in a petri dish together with 20 wheat grains, inhibited the germination of the latter completely, while two seeds of the sweet variety, containing only a trace of amygdalin had no effect and ten seeds depressed the g.i. only to 85% (148). The HCN in this case may be set free by the action of enzymes contained in the

wheat grains, since it has been shown (85) that traces of HCN are freed from pure amygdalin by germinating test-seeds which apparently contain enzymes capable of hydrolizing amygdalin. The same effect as with bitter almond seeds is obtained with Aqua Amygdalorum amarum.

When bitter almonds are crushed their inhibitory effect is much stronger, and even only two crushed sweet almonds have a slight inhibitory effect because then the emulsin, which in the whole seeds is localized in separate cells, comes into direct contact with the amygdalin. This can be proved by the usual HCN-reaction which gives negative results with whole almonds but a strong reaction after one minute when crushed almonds are used (55). The same has been reported for *Prunus* seeds (85) where the whole seeds are ineffective, whereas the crushed embryos inhibit germination completely. When the seeds are washed for three days before being crushed, the inhibition disappears completely, an observation which in many cases may explain the fact that germination inhibitors are removed by washing or leaching the seeds for some time.

In *Crataegus* seeds HCN was liberated just before germination (39). This may indicate that the amygdalin, usually hydrolysed very slowly, was broken down very rapidly toward the end of the period of after-ripening and the HCN liberated from the tissue; then germination processes, once poisoned by HCN, were freed to function normally (140).

Ammonia

The water extract from sugar-beet seed balls inhibits or retards the germination of sugar beet and many other seeds (38, 45, 46, 135, 136) and is toxic to germinating seeds and seedlings (135). It has been stated (38) that this effect is due principally to the osmotic pressure of the extract caused by its inorganic constituents. But there are volatile substances present in the non-decolorized extracts which also cause inhibition (38). Stout and Tolman (135) found that ammonia, released by enzymes from the nitrogenous compounds of the extract, is involved. This is proved by the following facts:

(*a*) The chemical test shows that during germination ammonia is liberated from the seed-ball extracts (135, 136).

(*b*) The quantity liberated is 0.3–0.4 mg of free ammonia per cubic centimeter of extract. This is enough to account for the inhibiting and toxic effect of the extract, since in controls the same amount of ammonia, liberated from other sources, had exactly the same effect (135). Other authors also report the highly toxic effect of ammonia which is even more injurious to germination than HCN (8). This agrees with the records of some authors (103) that ammonia in a concentration of 1 : 24,000 hampers the germination of *Vicia Faba,* in 1 : 20,000 that of *Zea Mays.*

The hydrolytic agent responsible for the freeing of ammonia is present in sugar beet seed extracts and intact germinating seeds. Lettuce seed extracts also contain some hydrolyzing agent freeing ammonia. This offers an explanation for the germination inhibition observed when lettuce seeds are successively germinated on the same substratum (125).

Ethylene

Ethylene is liberated by many fruits, *e.g.,* apples and pears (33, 34, 106), as well as by whole plants. The amounts produced are fairly considerable (1.78 ml to 11.38 ml per kg of apples per 24 hrs (58)) and suffice to cause a very pronounced growth and germination inhibition.

Seeds of different species were put beneath jars of six to eight liter capacity together with two or three ripe apples. Germination of the seeds was completely inhibited or retarded (74, 75). The suggestion put forward (79) that the volatile inhibitor of tomato juice might be ethylene could not be proved. This volatile inhibitor is not formed when disinfectants are added and is developed only by the action of microorganisms (Orig.).

Mustard Oils

Observations and experiments with the strong inhibitors contained in different plants belonging to the Cruciferae support the suspicion that mustard oils are the inhibiting substances in such cases. This supposition was based on the following facts:

(*a*) The fruit of *Sinapis alba* is composed of two valves and a beak. The valves are dehiscent, and the beak bears one seed which remains enclosed within it. This seed does not germinate so long as it is within the beak. When taken out, it germinates immedi-

ately. Valves and beak have been shown to contain strong inhibitors (133, 165). The same holds true for *Sinapis arvensis* (148), *Brassica nigra* and other cruciferous fruits (133, 165). The inhibitor is volatile and can be partly isolated by steam distillation (148).

(*b*) Fifty whole seeds of *Brassica nigra,* placed in petri dishes, lowered the g.i. of 50 wheat grains in the same dishes to 23%. When the seeds were ground the g.i. of the wheat grains was only 6% (Orig.). The inhibitor set free from ground seeds is volatile.

(*c*) The juice of horseradish, radish and kohlrabi roots contains a strong volatile inhibitor (Orig.).

Now it is well known that mustard oils, mostly allyl-isothiocyanate (CH_2=CH—CH_2—N=C=S) and β-phenethyl-isothiocyanate (C_6H_5—CH_2—CH_2—N=C=S), are very common in all organs of plants belonging to the Cruciferae, especially in the genera *Brassica* and *Sinapis.* Seeds of *Brassica nigra* contain 0.7% of allyl-isothiocyanate which is extracted from them by steam distillation and sold as Oleum Sinapis (92% to 95% allyl-isothiocyanate). Germination experiments using this oil proved it to be a strong volatile inhibitor, inhibiting in the same concentration as the inhibitor of *Brassica* seeds (148) and adsorbed by charcoal in the same way. In the seeds the glucoside sinapin, not the free mustard oil, is hydrolyzed by myosin to d-glucose, potassium hyposulphat and allyl-isothiocyanate. This explains the fact mentioned under (*b*). β-phenethyl-isothiocyanate is very similar in effect to that of allyl-isothiocyanate. It is most interesting that seedlings of *Raphanus sativus, Brassica alba, B. oleracea* var. *capitata,* var. *gemmifera* and var. *caulocarpa* give off mustard oil vapors which kill potato test plants (34). All together, it is certain that natural allyl- and β-phenethly-isothiocyanate (and apparently other mustard oils belonging to the same chemical group) are germination inhibitors.

Organic Acids

Blastokolin is the germination-inhibiting substance postulated by Köckemann (74, 75) as being in the fruit flesh of apples, but Tetjurew (139) came to the conclusion that "blastokolin is nothing else than a mixture of malic and citric acid". These two acids are the most common in fruit juices and plant sap. The fruit juice

of tomatoes contains 0.48% malic acid; of *Vitis vinifera,* 1.16%; of *Sambucus nigra,* 1.1%; of *Vaccinium Myrtillus,* 0.85% (147). The organic acid content is especially high in citrus fruit (5% to 6%).

While some inhibiting influence is exerted by these acids on germination through their control of pH, it is mainly by their intrinsic natures as specific acids that they are inhibitors. This is true also for other naturally occurring acids (43). Evidence for this lies in the fact that the pH of the lowest concentration causing complete inhibition is not significantly different from the pH of the highest concentration permitting normal germination. Such figures are as follows: citric acid, 1/100 (pH 2.39) and 1/2,000 (pH 2.93); malic acid, 1/200 (pH 2.57) and 1/1,000 (pH 2.87); salicylic acid, 1/1,200 (pH 2.45) and 1/6,000 (pH 3.30); acetic acid, 1/1,000 (pH 3.30) and 1/6,000 (pH 4.4); crotonic acid, 1/1,000 (pH 3.2) and 1/4,000 (pH 4.2). Since crotonic and acetic acids still inhibit completely at a pH of 3.2 or 3.3, whereas citric and malic acids do not inhibit any longer at a pH of 2.81 to 2.93, the pH cannot be held responsible for the inhibition caused by these two organic acids. There is, however, some influence of the pH in each case, for nearly always the inhibition is weaker when solutions of neutral salts of the different acids are used (43, 139).

The lowest concentrations for complete inhibition show that wherever the free acid content of inhibiting juices or sap reaches 1% for citric acid, 0.5% for malic acid, 0.2% for tartaric acid, 0.1% for acetic and crotonic acid, and 0.09% for salicylic acid, the inhibition is caused at least partly by these acids wholly apart from their influence on the pH.

Since different acids are always mixed in juices and sap, the synergistic effect of them must be taken into consideration too. Whereas a 1/2,000 dilution of citric or of malic acid alone causes no inhibition, the g.i. in a mixture of equal parts of these acids at this dilution is only 65% (43). The synergistic effect of a three-acid mixture is still more pronounced. A mixture of 0.025% citric, 0.025% tartaric and 0.01% salicylic acid (total acids 0.06%) exerts a much stronger effect than each acid alone in a concentration of 0.06% (43).

Besides the acids cited, cinnamic acid (trans-form) and some of its derivatives seem to be of special importance as inhibitors.

Trans-cinnamic acid formed by guayule plants is a growth- (12) and germination-inhibitor (130). Sodium cinnamate and dihydrocinnamic acid inhibit germination completely, even in concentrations of 0.01 mol. (124). Caffeic acid (3,4-hydroxy-cinnamic acid) and ferulic acid (3-methoxy-4-hydroxy-cinnamic acid) have been isolated from tomato juice (1) and are at least partly responsible for its germination-inhibiting action. Traumatic acid, the plant wound-hormone, is also a germination inhibitor (40).

Unsaturated Lactones

The fruit of *Sorbus aucuparia* contains a strongly inhibiting substance, parasorbic acid ($C_6H_8O_2$), which inhibits germination of *Lepidium* seeds in dilution of 1 : 1,000 (calculated on the pure substance) and gives at 1 : 10,000, 10% to 80% germination (82). It is present in the berries of the mountain ash in a concentration of at least 1 : 1,000 (Kjaer, according to 88), and belongs chemically to the unsaturated lactones.

Anemonin ($C_{10}H_8O_4$), another unsaturated lactone, found in various members of the Ranunculaceae, is a germination inhibitor, too (Kjaer, according to 88).

Coumarin ($C_6H_4OCOCH:CH$), another naturally occurring and widely distributed unsaturated lactone, is a strong inhibitor and was already mentioned by Sigmund (129, 130). In concentrations of 1 : 10,000 only 2% of the seeds germinate (82). On wheat coumarin is less effective, since the lowest concentration for complete inhibition is 1 : 8,000 (Orig.), proving that coumarin is one of the most potent inhibitors known.

It is most interesting that coumarin brings about an induced dormancy in non-dormant lettuce seeds (109). After coumarin treatment the seeds react to dormancy-breaking agents, *e.g.*, thiourea, exactly as seeds do which are naturally dormant. It is possible, therefore, that coumarin is the natural germination inhibitor of lettuce seeds (125, 126). The inhibitor or inhibitors of tomato juice are, according to Veldstra (153), unsaturated lactones, too. He writes: "We isolated from tomato juice a liquid lactone and a crystalline acid, both of them inhibiting seed germination and according to their chemical properties belonging to the discussed series", *i.e.*, unsaturated lactones. No further evidence has yet been published.

Aldehydes

As far back as 1910 Mazé (101) reported that unripe grains of corn and unripe seeds of peas containing 50% to 60% of water do not germinate, but do so when dried artificially. This is not due to the relatively great quantity of water contained in the unripe grains, but to the presence of an inhibitor, for ripe seeds do not germinate in extracts of the unripe ones. This inhibition is attributed to acetaldehyde which is present in unripe (0.1 mg per 20 gr. of corn) and missing in ripe seeds. In germination experiments the same concentration of pure aldehyde inhibited germination. The same author (100) is of the opinion that seeds placed under water for some time lose their vitality because acetaldehyde accumulates.

Benzaldehyde (C_6H_5CHO), which is formed during hydrolysis of amygdalin, is a strong inhibitor (129, 130 and Orig.). The lowest concentration of complete inhibition is 1/2,000 to 1/3,000; of incipient inhibition, 1/8,000 to 1/10,000 (Orig.). Its vapours, too, are very inhibiting; 0.0006% by volume still inhibits germination of wheat grains completely. Benzaldehyde undoubtedly participates in the inhibition brought about by bitter almonds.

Salicylaldehyde (O—HOC_6H_4CHO) is an even slightly stronger inhibitor which finds its explanation in the fact that the OH group is also an active inhibiting group.

Citral $(CH_2—\underset{\displaystyle CH_3}{\underset{|}{C}}=(CH_2)_3—\underset{\displaystyle CH_3}{\underset{|}{C}}=CH—CHO)$

present in lemon grass oil inhibits completely at 1/500 (130); its limit of complete inhibition is 1/1,000 to 1/2,000 (Orig.).

The related linalool

$(CH_2—\underset{\displaystyle CH_3}{\underset{|}{C}}=CH—(CH_2)_3—\underset{\displaystyle CH_3}{\underset{|}{C}}—C(OH)—CH—CH_2)$

has at 1/500 only a very slight inhibiting action (g.i. 62% (130)), showing that the CHO group is really responsible for the inhibition and appears to be active practically without any influence of the neighbouring groups.

Cianamalaldehyde (C_6H_5—CH=CH—CHO), too, is a very strong inhibitor (130), much more effective than cinnamic acid.

The same effect of the CHO-group is noteworthy in comparing the activity of anisicaldehyde (p-methoxybenzolaldehyde (p-$CH_3OC_6H_4CHO$) and anisic acid (p-methoxybenzoacid-p-$CH_3OC_6H_4COOH$) where in 1/500 dilutions the aldehyde depresses the g.i. to 8%, the acid to 39% (130). Other examples are: 1/200 mol crotonic acid, g.i. 98%; crotonaldehyde, 0%; salicylic acid 1/200 mol, g.i. 54%; salicylic aldehyde, 0% (Orig.).

Though from chemical considerations one should expect a strong inductive influence of the various constituents, it appears that as far as germination is concerned the specific nature of the isolated CHO group determines the extent of inhibition, while inductive and electromeric influences are of secondary importance.

Essential Oils

When the flavedo of a half or a whole lemon or orange peel is removed and put into a big petri dish in which a small petri dish is placed containing 50 wheat grains so that there is no direct contact between the wheat grains and the peel, the germination of the wheat grains is completely inhibited by the flavedo (Orig.). That the essential oil contained in the peel is the cause of this inhibition may be concluded for the following reasons:

(*a*) When the flavedo is peeled off and the oil glands are cut open by the peeling operation, the g.i. of 100 wheat grains is reduced to 18% by 20 cm^2 of peel used in the volatility experiment described above. If the peel is cut together with its albedo, and the glands remain uninjured as far as possible, the g.i. is 48%. When the peel is cut as in the first experiment but is washed thoroughly before being used, there is no inhibition at all (Orig.).

(*b*) When the essential oil of the peel is extracted by the usual steam distillation, the distillate inhibits strongly and the remaining peel loses its inhibiting influence (g.i. 90%, Orig.).

(*c*) The pure essential oil of lemon (Oleum Citri)—even when only traces are smeared on the inside of the cover of a petri dish—inhibits germination (148). All essential oils used (Oleum Coriandri, Rosmarini, Lavandulae, *etc.* (148)) show the same effect.

According to Sigmund (130), the descending order of inhibition is: Oil of cloves (2%), of peppermint (6%), of rosemary (6%), of

rose geranium (6%), of caraway (7%), of thyme (18%), of fennel (21%), of lavender (26%), of rue (34%), of Eucalyptus (51%), of anise (32%). These percentages designate the g.i. on the third day.

Different fruits, seeds and leaves containing essential oils show the same inhibiting effect, even when not in contact with the test seeds. With the fruit of *Foeniculum vulgare* (148) and its essential oil (Oleum Foeniculi), exactly the same experiments as described for lemon peel can be made (148). One half gram of rosemary leaves used in the volatility experiment give a g.i. of 44% (Orig.).

The growth-inhibiting and killing effect of essential oils on plants has long been known (26, 61), and the plants producing the oils are much more resistant to their own oils than are other species. *Brassica* was killed by *Dictamnus* vapours after four days, whereas *Dictamnus* remained intact for 12 days when branches of the two plants were placed beneath the same bell jar (61). Vapours of essential oils are more effective in this respect than liquid oils or solutions. The same may be observed for germination inhibition (Orig.).

Since the groups of the so-called "essential oils" are heterogenous from a chemical point of view, we do not know which constituents are responsible for the inhibition. There are only some indications as to the active components (130):

(*a*) Aromatic and alicyclic hydrocarbons (styrene, p-cymene, d- and e-pinene, d- and e-limonene), alcohols (borneol, linalool, menthol, terpinhydrate) and acids are apparently not responsible for the inhibition, for all such substances did not inhibit germination at all or did so only very slightly. The only exception was terpineol which inhibited more strongly than the other compounds.

(*b*) Aldehydes (benzolaldehyde, salicylic aldehyde, citral, cinnamal-aldehyde, anisic-aldehyde), phenols (thymol, carvacrol, apiol, safrol) and ketones (carvone, camphor, fenchone, eucalyptol), which are present in many essential oils, perhaps in all, are strong inhibitors and may be responsible for the inhibition.

Aldehydes and phenols especially are to be considered the main active components, described as far back as 1893 as Ermüdungsstoffe (119), *i.e.*, metabolites which inhibit or retard growth, protoplasmic movement and other processes. Thymol, carvacrol, cinna-

mal-aldehyde and salicylic aldehyde are cited specially. Their "plasmatiring" action is explained as that of substitute poisons, a very modern idea indeed.

Alkaloids

Oppenheimer (110, 111) long ago assumed that the inhibition of tobacco seeds, when put near their seed capsules or leaves, might be caused by the alkaloids contained in the latter. All seeds and fruits known for their high alkaloid content are strong germination inhibitors (148), and it can be proved that the alkaloids are the main if not the only cause of inhibition in these cases. The reasons are:

(*a*) When the alkaloids are removed by suitable extraction methods (the alkaloid-containing extracts inhibit), the inhibition of the seeds from which the alkaloid was removed is greatly diminished (148).

(*b*) Pure alkaloids in concentrations much weaker than those in which they are contained in fruits and seeds, exert the same inhibition as the fruits and seeds themselves (148). (Coffee seeds contain 0.3% to 2.36% caffein; coca leaves, 0.25% to 0.6% cocain; seeds of *Strychnos nux vomica,* 1.12% to 2.23% strychnin and 2.3% to 3.9% total alkaloids; seeds of *Physostigma venenosum,* 0.17% physostygmin; cocoa seeds 1.05% to 2.34% theobromin and 0.05% to 0.36% caffein (147). (Compare the germination experiments made with 1/200 solutions of pure alkaloids!).

(*c*) In the genus *Trigonella,* only the alkaloid-containing species, *Foenum graecum,* inhibits germination. Only 20 seeds suffice. The seeds of the closely related species, *T. Kotschyi* and *T. monspeliaca,* which do not contain alkaloids, do not inhibit, even when 100 seeds are used (148).

Of all alkaloids investigated (43, 128, 148), only the following inhibit. (The percentage figures indicate the g.i. of wheat grains in 1/200 dilutions):

(*a*) Strong inhibitors: Cocain (0%), physostigmin (0%), caffein (2.5%), chinin (3.5%), cinchonin, cinchonidin, tropa acid, strychnin (25%), berberin and codein (35%).

(*b*) Weak inhibitors: Narkotin and scopolamin (58%), emetine (65%), papaverin (65%), ephedrin (73%), piperine and atropine (81%).

When more diluted, most alkaloids stimulate germination, as pointed out by many authors (see 37, where older literature is cited) who sometimes even write about the hormonal action of alkaloids.

CORRELATION BETWEEN INHIBITORY ACTIVITY AND CHEMICAL STRUCTURE OF THE INHIBITORS

Only the first steps have been taken to elucidate the relation between inhibitory effect and chemical constitution of inhibitors.

Mustard Oils

The group $-S-C\equiv N$ or $-N=C=S$ is four to five times more effective than the group $-C\equiv N$ or $-N=C$, since the limit of complete germination inhibition lies for allylcyanid and allylisocyanid around 1/1,500, for allylisothiocyanate and allylthiocyanate around 1/5,000–1/6,000 (Orig.). The same results are obtained with phenylisothiocyanate and phenylcyanid.

There is no difference between $-S-C\equiv N$ and $-N=C=S$ (Orig.).

The type of radical attached to the active group changes the inhibitory effect of the compound. The order of decreasing activity is: phenyl, allyl, ethyl (Orig.). The fungicidal effectiveness has shown another order of decreasing toxicity: allyl, phenyl, methyl, ethyl (157).

The introduction of halogen atoms enhances the inhibitory effect (Orig.).

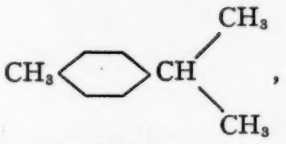
Phenols and Organic Acids

With phenols the inhibitory effect decreases generally with the increasing number of OH groups, *e.g.*, phenol, resorcinol, phloroglucinol. The results of Sigmund (130), obtained with *Vicia* and *Rapa* as test seeds, might indicate a position effect of the OH-group. The g.i. figures for *Vicia* are: catechol (orthodioxy-benzol) 2.1%, resorcinol (metadioxybenzol) 22%, hydroquinone (paradioxybenzol) 96%, pyrogallol (vicinal trioxybenzol) 21%, phloroglucinol (symmetric trioxybenzol) 93% (1/40 mol. solution, 5th day of germination).

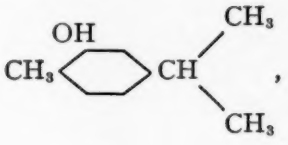
In quite another way, too, it can be shown that the inhibition caused by phenolic compounds is a function of the active OH group

and is largely independent of the nature of the structural nucleus to which it is attached.

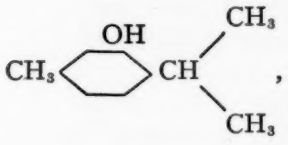
Cymol,



in 0.2% solution has a g.i. of 81%;
carvacrol,



and thymol,



of 0% (130).

With aromatic carboxylic acids the inhibitory effect decreases with the increasing number of carboxylic groups.

The introduction of an OH group enhances the inhibition (1/200 mol. of benzoic acid 17%, salicylic acid 0%). If more OH groups are introduced the inhibition decreases (gallic acid 9.6%, protocatechuic acid 39%).

When there are side chains a double bond increases the activity (mentioned later for tropa and atropa acids). The same holds true for aliphatic carboxylic acids (129, 130, Orig.). Halogenation of the benzene ring increases the inhibiting action (benzoic acid 1/200 mol. germination inhibition of 21%, orthomonochlorbenzoic acid 0%).

Alkaloids

This group is much too complicated to permit ascertaining any relationship between structure and function. A few interesting facts should be mentioned, however. Atropin does not inhibit or does so only very weakly (128). From its cracking products atropa acid is a very strong inhibitor, tropa acid inhibits less, and tropin shows less inhibition than atropin (128). The increased

activity of atropa acid in comparison to that of tropa acid is perhaps due to the presence of a double bond. The related compounds chinin, cinchonin and cinchonidin are all strong inhibitors.

PHYSIOLOGICAL ACTION OF THE INHIBITORS

Inhibition and Stimulation

All authors who have tried to elucidate the physiological action of the different juices and extracts containing inhibitors have found that inhibition is accompanied by a stimulation of germination and growth. At sufficiently high dilution, the inhibition of tomato juice is replaced by stimulation. This stimulation starts at a dilution of 1/8, reaches its peak at a dilution of 1/60 to 1/250 and disappears at 1/1,000 (79). Tobacco seeds germinated on tobacco seed capsules are at first strongly inhibited, but show a considerable stimulation after 20 days (111). The water extract of wheat grains stimulates after an initial inhibition lasting three or four days (107). Different seeds placed for some time into *Beta* seed ball extracts and then into water show a three- to four-fold increased germination and growth velocity (47). Fruits of *Helianthus, Avena* and *Triticum* excrete, together with an inhibitor, a non-specific substance stimulating germination (123).

There are three ways to explain the relation between inhibition and stimulation:

(*a*) The same substance inhibits in high and stimulates in low concentrations.

This is in agreement with the well known fact that hormones and poisons inhibit at high and stimulate at low concentrations. The action of different dilutions of tomato juice seems to confirm this supposition.

(*b*) Inhibition and stimulation are caused by two different substances.

The stimulating action of tomato juice is much more pronounced in dialysed than in undialysed juices, and the stronger the inhibition in undiluted juice, the less marked is the stimulation at high dilution (79). In wheat extracts the inhibiting action is eliminated by heating, but the stimulating factor remains (107). These facts point to the existence of two different substances which are present at the same time in the undiluted juices. The inhibitor is more sensitive to dilution than the stimulator whose action is concealed

by the inhibitor in low dilutions and is manifest only at high dilution where the inhibitor is rendered ineffective.

(*c*) The inhibitor is transformed into a stimulator by a slight chemical change during germination.

In *Helianthus* the dry fruit in all its parts contains only inhibitors. When the different organs are isolated from seeds after imbibition of six hours, the plumule and radicle stimulate, the cotyledons and fruit coat inhibit initially, but after some time the inhibiting action of the cotyledons is reversed into stimulation. After an imbibition of 15 to 18 hours, all organs stimulate besides the fruit coat which shows an initial inhibition (123).

Ruge draws from these experiments the conclusion that during germination the inhibitors are transformed into stimulators as long as the different fruit parts are in organic contact. This transformation does not take place and the inhibitors remain unchanged when the different organs are extracted from dry fruits and imbibed separately. Ruge, together with other authors (150, 151), is of the opinion that auxins are not involved. They developed an interesting theory about the formation of inhibitors and transformation of them into stimulators. The following steps are supposedly involved:

(*a*) Hydrolysis of a nitril-glucoside of the amygdalin type and formation of HCN which is a known inhibitor.

(*b*) Addition of elementary sulphur to the HCN molecule by action of rhodanase and formation of HSCN which, according to Gemeinhard (55), is a stimulator.

We may mention here the interesting theory of Veldstra (152) concerning growth stimulators and inhibitors. He has shown how the same substance can inhibit or stimulate in different concentrations and produce changes in the structure of plasmic membranes. This may be analogous to our germination inhibitors and stimulators which—like the auxin in Veldstra's theory—could swell or condense the plasmic membrane, regulate permeability and hamper or stimulate different processes necessary for normal germination.

Germination and Growth Inhibition

Germination- and growth-inhibition are nearly always associated with one another. When seeds in different stages of germination are transferred from water to tomato juice, their growth is

inhibited. In a very advanced stage they continue to sprout, but the radicles turn black and degenerate (79). Generally the roots are much more sensitive to the inhibition than the coleoptile, plumule or young sprouts. The same holds true for wheat grain extract and its inhibitor (107). Froeschel (47) states, concerning the *Beta* seed inhibitor, that germination inhibition is only one aspect of the inhibition problem and that the second aspect is the considerable inhibition and retardation of growth. Barley extract inhibits germination and growth (83). *Beta* seed-ball extract inhibits considerably all cell elongation in the *Avena* coleoptile cylinder test (38). In the *Avena* curvature test, tomato juice, parasorbic acid and anemonin retard growth (88). Different alkaloids (caffein < chinin < strychnin < atropin < ephedrin < codein < nicotin) have the same effect (43). Growth inhibition is still apparent in dilutions in which there is no longer any trace of germination inhibition. Certain leaf saps must be diluted to one-tenth of their normal strength to eliminate germination inhibition, but to 1/25 or 1/50 to eliminate growth inhibition. The respective figures for coumarin are 1/40,000 and 1/100,000 (158 and Orig.); for phenylisothiocyanate, 1/50,000 and 1/80,000 (Orig.).

All these facts may indicate that germination inhibition is only an inhibition of the first phases of growth.

There are some observations which do not fit into this picture, namely, that some growth inhibitors are inactive upon germination (96), and that sometimes the same substance acts differently on growth and germination (96). Today the experimental data are too scant to decide this question. "The identity of the inhibition of growth and germination has never been proved, although it has tacitly been assumed by several authors" (38).

Inhibitors and Auxins

Many authors (31, 84, 105, 108, 120) have found auxins in seeds, and some (84, 105) have shown that the auxin content of seeds decreases during germination.

If the auxin present in seeds were to function as a "germination hormone", the action of inhibitors could be explained by auxin destruction or inactivation. But, can auxins be considered as germination hormones? Veh and Söding (151) answer this question in the negative. The germination inhibition caused by apple

seed endosperm can be explained neither by inactivation of its auxin, since the endosperm contains active auxin, nor by any inhibiting influence of the auxin, for the quantities contained therein are too small for that. Furthermore nearly all authors have found that different growth hormones inhibit germination only at higher concentrations than are found naturally in plants and are of no effect in lower concentration (review in 6, 7). Only when seeds were used which normally exhibit some delay in germination or old seeds whose germination capacity was decreased, was there some stimulation of the treated seeds (2, 6, 7, 86).

There are a number of facts supporting the second supposition that inhibitors destroy or inactivate growth hormones or lower the response of cells to them. Beet-seed extract, for instance, applied together with heteroauxin in the *Avena* coleoptile cylinder test decreased the activity of the latter (38).

Mixtures of growth hormones, on one hand, and tomato juice, parasorbic acid and anemonin, on the other, when applied in the *Avena* curvature test, suppress or decrease the curvature of the coleoptile (88). This explains the shape of the curves produced by plant extracts which apparently contain a growth-retarding substance together with a growth-promoting one (3-indol-acetaldehyde). In tomato juice Larsen (87) observed three different growth inhibitors and assumed that one of them is identical with the acid germination inhibitor present in this juice, a supposition supported by Juel (69). He found that the acid extract of tomato juice inhibits growth of coleoptiles and roots. This inhibitor does not destroy the auxins but counterbalances their activity. Since Konis (80) found germination inhibitors in nearly all leaf sap, and other authors (53, 92, 134, 154, 155) have noted growth inhibitors in the extracts of different parts of numerous plants which act as antagonists to growth-promoters, there is a case for the identity of germination and growth inhibitors.

Caffein applied together with heteroauxin in the *Avena* test affects the activity of the latter very strongly (43). It is noteworthy that the growth inhibition is stronger when caffein and heteroauxin are applied together or one after the other than when caffein is used alone (43).

An interesting theory concerning HCN as inhibitor and heteroauxin as stimulator is proposed by Keil (72) for apple seeds. Ac-

cording to him, apple seeds do not germinate normally because HCN in the surroundings of the embryo narcotises all the cells and lowers their response to heteroauxin which is not attacked by HCN. When the HCN is removed, the seeds germinate, for then the "narcosis" of the cells to heteroauxin is abolished.

Effect of Inhibitors on Germination Capacity

When wheat grains are placed in tomato juice for varying lengths of time and then transferred to water, germination is retarded, even after only two hours in the juice. Submersion of 50 hours or more renders the grains incapable of germination (79). The same holds true of the *Beta*-inhibitor where seeds of *Antirrhinum* and *Medicago* are irretrievably damaged after seven days stay in the extract. The seeds of 11 other species germinated properly, even after a long stay in the extracts, when the inhibitor was removed by washing the seeds before their transfer to water.

Inhibition and Light

A very peculiar feature of inhibitors worthy of further elucidation is their reaction to light, even when the test seeds germinate in light and darkness alike. The inhibition of wheat grains caused by wheat extracts is stronger in light than in darkness (107). *Lepidium* and *Gypsophila* seeds are much more inhibited by *Beta* extracts in the presence of light. Darkness diminishes inhibition considerably (47). Decolorised *Beta* extracts, which apparently contain only inorganic salts as inhibitors, show the same light effect (38). Even with isotonic salt solutions the inhibition is much more pronounced in light (38). A similar effect is observed with seeds whose germination is normally influenced by light. Seed extracts of *Phacelia tanacetifolia,* a darkness-germinator, inhibit only in weak light and are ineffective in darkness (97, 114). Dormant lettuce seeds are 20° to 25° C-darkness germinators and do not germinate in light. Non-dormant seeds are made light-sensitive by treatment with coumarin, *i.e.,* when treated with coumarin in light or when light strikes the seeds after coumarin treatment, while the seeds are still wet, germination is inhibited.

There can be no doubt that the problem of seeds whose germination is influenced by light or darkness is closely related to the problem of inhibitors. There are many indications (*e.g.,* 54, 141)

that in light-germinators, *e.g., Chloris ciliata,* an inhibitor is formed during germination which is destroyed by light of a certain wavelength or by application of certain chemicals. The germination-stimulating influence of the light could be explained as "an inhibition of an inhibitor" (54).

With darkness germinators, *e.g., Phacelia tanacetifolia,* the inhibitor could be a photodynamic substance which inhibits only in the presence of light (97). We may mention in this connection that Metzner (104) found photodynamic fluorescent substances in many different seeds. It would be most valuable to follow this line of research, connecting the inhibitors with the highly interesting problem of radiation influence on germination.

There are two other very interesting angles to the problem of radiation influence on inhibitors. The growth hormone auxin-a-lactone is transformed to lumi-auxin-a-lactone under the influence of light. Lumi-auxin is supposed to be an unsaturated lactone, acting, like most unsaturated lactones, as a growth inhibitor (153). This is in harmony with the above cited seeds, where inhibitors are activated by light, and with coumarin- (an unsaturated lactone!) treated lettuce seeds, where germination is inhibited by light. Secondly, trans-cinnamic acid, formed by guayule plants (11, 12), is a strong growth-inhibitor (1 mg per liter is active), while cis-cinnamic acid is a growth hormone, and trans-cinnamic acid is transformed into cis-cinnamic acid by ultra-violet radiation (163). These facts offer a parallel to those seeds where light produces an "inhibition of an inhibitor".

Inhibition, Swelling, Respiration, Enzyme Activity

Inhibitors could function by hampering the water intake of seeds. All experiments in this direction (79, Orig.) proved that water intake is not influenced at all or is only very slightly depressed by inhibitors and is decidedly of no importance in explaining the action of germination inhibitors. There is in most cases an influence of the inhibitors on respiration. Respiration decreases about 10% in seeds treated with tomato juice (79). Barley seeds treated with their own inhibitor show much decreased respiration (83), but the respiration of *Beta* seeds soaked in the decolorised extract is not affected during the first four hours.

As far as hydrocyanic acid is concerned, the inhibition caused by

it is certainly related to the well known strong inhibiting effect of this substance on respiration.

Konis (79) reports that the addition of juice to diastase *in vitro* does not alter its activity, whereas other authors (83) report decreased diastase activity of barley grains treated with their own inhibitor.

Inhibition, Oxygen, Carbon Dioxide

The role of oxygen and carbon dioxide in inhibition was recently discussed by Thornton (see 140 for literature). Secondary dormancy, *i.e.,* delayed and inhibited germination, can be induced in many seeds by lowering the normal oxygen supply. This happens when the oxygen in the atmosphere surrounding the seeds is replaced by carbon dioxide or when the seed coats are impermeable to gases. Then the oxydizing system is greatly inhibited and intermediate products of partial anaerobic respiration accumulate, *e.g.,* acetaldehyde (101) and many other toxic substances. These bring about dormancy, *i.e.,* they inhibit germination (140 and literature cited therein). These presumably accumulated inhibiting substances tie up the question of induced dormancy with our problem of germination inhibitors.

Thornton tries to explain in the same way the known effects of high and low temperature on germination and the interruption of dormancy: "High temperature storage increases the dormancy as the hydrolyzing system remains more active than the oxidizing one. Low temperatures retard the accumulation of inhibitors and lead to germination".

This theory should be used as a working hypothesis in further experiments, for it associates inhibitors, respiration, oxygen, CO_2 and different temperatures with germination and dormancy. It is noteworthy in this respect that recent observations have recorded (66) that 2,4-D, one of the most potent artificial inhibitors, affects aerobic respiration. Barley seeds germinating in an atmosphere of oxygen after treatment with 2,4-D, cannot use the O_2 and respire in an anaerobic way, as do untreated seeds germinating under low O_2-pressure.

Inhibition and Permeability

Essential oils decrease the protoplasmic permeability of *Elodea* and *Spirogyra* (137), but after some time the permeability is in-

creased, so that water drops are excreted (Gigliogli, cited in 137). Later the cells die. It is possible that the inhibitory activity of essential oils is connected with their effect on permeability. Inasmuch as experimental data are scant, it would be desirable to pursue this line of research, especially since Veldstra (152) has put forward his interesting theory.

BIOLOGICAL FUNCTION OF INHIBITORS

The main biological functions of germination inhibitors are prevention, by self-inhibition, of premature germination; extension of the germination period over a long time; and suppression of germination of other species in the surrounding of the plants producing inhibitors.

Prevention of Premature Germination

If seeds were to germinate inside the fruits that contain them, one of their main biological functions—dispersal of the species over a wide area—could not be fulfilled. Such premature germination is prevented by the inhibitors within the seeds themselves. Another function would be the prevention of germination of seeds not yet ripe, if Mazé's experiments (101) can be affirmed.

Extension of the Germination Period Over a Long Time

In cases such as *Sinapis,* some of the seeds remain enclosed in the fruits and do not germinate, while other seeds fall out and germinate immediately. The enclosed seeds germinate only after the inhibitor contained in the fruit coats has been washed out by rain or the fruit coat has been destroyed by the action of microorganisms. This means that the germination of the seeds produced in one year is protracted over at least two years. If conditions are not favourable for such species during one year, germination the second year is assured by the reserve of germinable seeds remaining for the following year. The phenomenon of weeds reappearing in a field after a lapse of time may be explained in such a way.

Suppression of Germination and Growth of Other Species

Fröschel and Funke (48, 49) and Ullman (148) have shown that seeds containing inhibitors inhibit germination of other seeds when placed with them in pots containing soil or when planted directly in soil. *Agrostemma Githago* seeds do not germinate and the

seedlings do not develop in soil treated with *Beta* seed extract. Wheat grains do not germinate when planted with *Viola* seeds. *Melandrium* and rye grass do not develop when *Beta* seeds are present in the same pot. Most seeds containing essential oils, alkaloids or glucosides inhibit germination and development of other seeds which may be in their vicinity. Wheat and rye grains suppress the germination of weed seeds, such as *Anthemis arvensis* and *Matricaria inodora,* when planted in the same pot (116). The phytosociological importance of this phenomenon has been pointed out by some authors (48, 49, 148).

Germination and growth inhibitors are not restricted to seeds and fruits, but are secreted also by leaves and roots. Taking as a starting point the fact that parks of *Robinia pseudoacacia* are nearly void of all other vegetation, Waks (156) has shown that the bark and wood of *Robinia* contain substances hindering the growth of barley. Leaves of *Artemisia Absinthium* excrete ethereal oils and the alkaloid absinthin which inhibits the development of seedlings of *Foeniculum vulgare* and other species within a distance of one meter (10, 51). In one case (*Levisticum officinale*) the seedlings were even killed by the secretions of the absinthe plants themselves (51). Fresh leaves of *Artemisia Absinthium,* when dug into the soil, reduced the percentage of germination of a number of species (51).

Since the number of reports of plants excreting substances inhibiting germination and toxic to growth (11, 12, 106) is increasing, this phenomenon may well be of considerable biological and phytosociological importance.

INHIBITION, ANTIBIOTICS, MUTAGENOUS SUBSTANCES, PHYTONCIDES

Some inhibiting juices and most of the chemical groups to which inhibitors belong are known also as "antibiotics", "mutagenes", "phytoncides" or "insecticides", or are used in medicine because of their special physiological activities. Tomato juice, for example, kills *Paramaecium caudatum* (113). Space permits us to consider in this respect only mustard oils, unsaturated lactones, essential oils and alkaloids.

Mustard Oils

As far back as 1900 Beijerinck (9) found that "Benzylsenfoel" inhibits the growth of *Saccharomyces.* Seeds of *Brassica oleracea*

contain an antibiotic principle belonging to the mustard oils (94), and the resistance of crucifers to clubroot disease (*Plasmodiophora brassicae*) is attributed to their content of such oils (122), a supposition supported by experiments showing the fungicidal activity of allylisothiocyanate and β-phenethylisothiocyanate (64, 65). The mustard oils and their vapours are highly toxic to different fungi in concentrations as low as 10 p.p.m. (115, 157, *etc.*). Vapours from crushed garlic show bacteriocidal effects (98, 159) which are attributed to allicin (allyl-$\overset{\overset{S}{\|}}{}$-S-allyl) (19, 20, 21). Allylthiocyanate is a "mutagene" with *Drosophila,* producing mutations (3). Treatment with allylisothiocyanate vapours causes structural changes in somatic chromosomes of *Drosophila* (132).

Kolle and co-workers (77), long before the Russian authors invented their "phytoncides", described the strong inhibiting action of vapours of different mustard oils in garlic, onion, radish and horseradish on animal tissue cultures and on bacteria. Microglia cells, for instance, are killed after an exposure of only two minutes to vapours of allylmustard oil given off by a 1 : 1,000 solution (77). Vapours are inhibitory at much greater dilution than are solutions (77), as is evidenced by the effects of essential oils (61 and Orig.).

Volatile "phytoncides" found in bulbs of onion and garlic have bacteriocidal and protistocidal properties, are lethal to yeast and eggs of mollusks and frogs, and have been used in medicine for keeping wounds clean and for stimulating regeneration of tissues (142, 143, 146, *etc.*). It seems certain that the phytoncides from garlic and onion are mustard oils. Garlic and onion have been used since time immemorial in folk medicine as disinfectants in stomach and intestinal infection.

Unsaturated Lactones

The antibiotics patulin, expansin, penicillic acid, clavonin, *etc.*, and perhaps penicillin itself, contain an unsaturated lactone ring (22, 82, 153, 158). Anemonin and protoanemonin are antibacterial agents (5). Cardiac glucosides, *e.g.,* digoxigenin, gitoxigenin and the very potent strophantidin, have an unsaturated lactone ring. Besides their physiological activity on the heart action

they are antibiotic, delaying the souring of milk, and show sterilising properties (153).

Bufotalin and bufotoxin, the most important toad poisons, and many insecticides (153) are unsaturated lactones.

Parasorbic acid and related unsaturated lactones inhibit growth of bacteria, epithelial tissue and fibroblasts (82). This action is attributed to their interference with cellular proliferation because of their reactivity with SH groups essential to enzyme function (59). This mechanism could explain their germination-inhibiting action as well.

Medawar and co-workers (102) isolated from malt extracts a differential growth inhibitor, apparently the unsaturated lactone of δ = oxy = $\alpha\beta$ hexenoic acid.

Many coumarin derivatives act as anesthetics (153), and perhaps the anti-coagulant action of di-coumarol, which is also an antibiotic (57), is based on its being an unsaturated lactone.

Essential Oils

Essential oils have long been known and used as disinfectants because of their antibiotic action, especially against bacteria (18, 23). Focke (44) stated as far back as 1881 that plants producing essential oils are not attacked by parasitic fungi. Glands containing essential oils are protected against putrefaction. Leaves of *Rosmarinus, Salvia officinalis* and *Mentha viridis* are completely destroyed after being placed in pond water for four months, but the glands are left intact (36). Essential oils are very toxic to man and other animals. With this fact as a basis, Detto (36) believes that the biological function of essential oils lies in the protection they give plants against being eaten by animals. Such oils are "phytoncides", too, for different Russian authors (142, 143, 146) have found a strong bacteriocidal and protistocidal action in those of orange, lemon, mandarine leaves, *etc.* In their opinion "phytoncides" are not identical with essential oils but are components or derivatives of them.

Alkaloids

Drugs containing alkaloids are the oldest poisons, remedies and stimulants known to man, used even in prehistoric times (147). All of them are known for their outstanding physiological activity

which is based partly on their antibiotic properties (chinin), partly on the inhibition and poisoning of important physiological processes. Only one example, colchicine, need be cited, the modern means of inducing artificial polyploidy. It arrests spindle formation in animal and plant cells and inhibits separation of sister chromosomes in plant cells, slowing down metabolic processes at the same time (35).

Since many germination-inhibiting substances are, as indicated, effective as inhibitors of all kinds of physiological processes in plants and animals, it is probable that they all affect one or more very basic reactions common to all living organisms. The mechanism affected may be of the kind assumed by Veldstra, *i.e.*, condensation or dilation of the plasmic membrane, which would change the permeability, or it may be an attack on some steps of the respirational system, or it may be a substitution reaction of the type supposed by Cavallito and Haskell (22). One thing is sure—in tackling the problem of germination inhibitors and of inhibitors in general, one touches a basic biological phenomenon. Further research into this really fascinating problem, which was already known to Albertus Magnus (76), is certain to yield most interesting results.

SUMMARY

The presence of germination-inhibiting substances in plants seems to be a wide-spread phenomenon. They occur in all parts of plants —in fruit pulp, fruit coats, endosperm, seed coat, embryo, leaves, bulbs and roots. They are non-specific in their effects.

Besides inhibitors, high osmotic pressure and acid pH are often partly responsible for the germination inhibition caused by sap, juices and extracts. The main known inhibitors are the following substances or belong to the following chemical groups: hydrogen cyanide, ammonia, ethylene, mustard oils, organic acids, unsaturated lactones, aldehydes, essential oils and alkaloids.

In some of these groups a relation between inhibiting activity and chemical structure of the inhibitor can be established.

Germination inhibition is nearly always accompanied by stimulation of germination. Sometimes inhibition and stimulation appear in different concentrations, sometimes one after the other in the same concentration.

Germination inhibitors seem to fulfill a biological function and must be taken into consideration as important factors in phytosociology.

There seems to be a relation between germination- and growth-inhibitors, antibiotics, mutagenes and phytoncides which may be based in common on some basic physiological process or processes.

ACKNOWLEDGMENT

My thanks are due to Dr. James Bonner, California Institute of Technology, who encouraged me to write this paper, and to my cooperators and friends, Dr. Y. Leibowitz, Dr. A. Katschalsky, Dr. E. Konis and R. Richter, for help in preparing the manuscript.

Thanks are due also to Dr. Fulling who undertook the difficult task of improving the English in my manuscript.

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ADDITION

After the manuscript was already in print the papers cited below have been published or came to my knowledge.

Based on the observation of Went (179) that some desert shrubs favour the growth of certain annuals whereas annuals very rarely are found in the vicinity of *Encelia farinosa*, Gray and Bonner (169) isolated a growth inhibitor from the leaves of this plant which is 3-aldehydo-4-methoxy acetophenone. This toxic substance may be leached out from the fallen *Encelia* leaves and cause inhibition of germination of seeds in the vicinity of *Encelia* plants.

The importance of inhibitors for plant sociology and agriculture was stressed already in 1907 (174). Schreiner and co-workers (174–178) report that many soils are unproductive because of the presence of toxic substances which in some cases are excreted by roots. From soil which by constant recropping with cowpeas was made infertile for this crop a substance could be extracted which inhibited the growth of cowpeas in the water culture (177).

This offers an explanation for the well known phenomenon of soil fatigue. The same author has been the first to report that cinnamic acid and coumarin are strong growth inhibitors (175).

Pickering (171, 172) formulated long before Molisch (106) the idea of "allelopathie" by stating that "the deleterious effect of one

growing plant on another is a general phenomenon" (171), and explains by toxin production the well known baleful effect of grass on fruit trees.

The bark of peach roots has to be added to the list of plant organs containing inhibitors (173). Hydrogen cyanide produced from amygdalin is most probably the inhibiting agent.

The toxic effect of *Juglans nigra* trees on certain plant associations is explained by excretion of juglone (5-hydroxy alpha-napthaquinone) (167).

Human saliva was reported to exert a germination inhibiting influence (180).

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EFFECTS OF MINOR ELEMENTS ON THE PHYSIOLOGY OF FUNGI

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INTRODUCTION

Numerous reports on the effects of metallic ions on the physiology of fungi have appeared during the nine year period (1939–47) since this subject was reviewed by Foster (41). It is evident that metallic ions play important roles in the production of many chemicals including the antibiotic substances by microorganisms, and some advance has been made in understanding the effects of metallic ions on fungal metabolism as a by-product of the recent intensive research on the mycological production of these compounds. However, there is still considerable ground to be covered before the specific roles of these metallic ions in the metabolic functions of the organisms is fully understood.

It has been decided to confine the material covered in this review nearly entirely to the heavy metals and to omit many of the studies concerned with the effects of the alkaline earth group. In order to integrate the literature in a satisfactory manner, it will be reviewed by organisms rather than by individual elements. While this method does not allow comparisons to be easily drawn, it seems more reasonable, since experimental conditions differ greatly in different laboratories, and generalizations are thus of somewhat limited value. Inasmuch as the actinomycetes apparently resemble fungi in the order of their requirements and their responses to the presence of metallic ions in the medium, they will be included in this discussion.[2] Neuberg (100) has included a general discussion on recent developments on the effects of metallic ions on yeast growth and metabolism, and more specific information is mentioned by Joslyn (59). Schopfer (128) and Stiles (150) have discussed the general subject of metallic ions with regard to the physiology of fungi in their recent books.

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[2] These organisms will not be included in a review on the effects of metallic ions on bacterial physiology (Dr. S. G. Knight, personal communication).

PURIFICATION OF MEDIA

In order to study the effects of specific ions on the physiology of an organism, it is advisable to have media which contain only known constituents. Most synthetic media contain sufficient contaminating quantities of these minor elements to be biologically important, and natural media have an abundance of them. It is necessary to remove these contaminants without affecting the other constituents of the medium. A number of methods have been proposed or have been rather widely used during the past few years and will be mentioned. Unfortunately it is not often possible to gauge the effectiveness of these methods in reducing the metallic content of the medium, since the quantities present before and after treatment are rarely stated. Stout and Arnon (153) have discussed methods for the purification of a number of medium constituents and criteria of purity.

Among the chemical methods recently described is that in which 8-hydroxyquinoline is used to form complexes with metallic ions (164, 175). Other related compounds may also be used (4, 5, 6, 19). The complex as well as the residual reagent is often removed from the medium by chloroform extraction before sterilization and inoculation. Other complex-forming agents and precipitants include ferrocyanide, ferricyanide (89) and calcium sulfide (20) which have been used in the purification of media in which molasses is a constituent (14, 15, 49, 68, 108, 135). Hutner (52, 53) has suggested the addition of certain hydroxy-organic acids to the medium to form complexes with metallic ions and apparently make them unavailable to microorganisms. This may also occur when organic acids, *e.g.*, citric, oxalic, tartic, are formed during the fermentation (111), and thus limit the effect of specific ions during fermentations. As can be seen this becomes of practical importance when such fermentations are operated on a large scale with several intermediate fermentation steps. The addition of peroxide (87) and other reagents (76) may also accomplish this biologically unavailable complex formation. Other specific relationships also have been reported, including histidine-cobalt (26, 110).

Treatment of the medium with cation-exchange materials has been suggested as a non-selective method of removing contaminating elements. Of course, this method is applicable only to the

non-ionic portion of the medium, and usually inorganic salts are added after treatment. The method is quite effective in reducing the quantities of metallic ions present (106, 165). It has been applied to synthetic media (105, 109, 110) as well as molasses solutions (60, 62, 117, 118, 135, 163, 169).

A practical method giving results similar to those obtained with the cation-exchange material was recently described by Shu and and Johnson (133b) who used aluminum hydroxide as a coprecipitating agent.

OBSERVATIONS ON THE EFFECTS OF METALLIC IONS ON GROWTH AND METABOLISM OF FUNGI

Ashbya gossypii. Wickerham *et al.* (166) have noted that an orange-yellow variant produced high yields of riboflavin when grown on unpurified media containing Fe.

Aspergillus clavatus. It has been observed that the use of raw sugar in place of technical glucose resulted in somewhat greater antibiotic potency (presumably patulin), and Waksman *et al.* (162) comment, "This suggests the possible function of some active agent or trace element present in the raw sugar".

Aspergillus flavus. Copper is apparently needed for growth and spore production (93). The poisonous effects of high concentrations of $MgSO_4$ on growth were counteracted by $HgCl_2$ (120). Pontovich (113) mentions that U radiation of this organism growing in a medium deficient in K showed that U prevented formation of melanins and stimulated riboflavin production.

Aspergillus fumigatus. Menzel *et al.* (88) found that the addition of Zn to Czapek-Dox medium completely suppressed the production of gliotoxin without affecting mold growth, and resulted in the formation of a red pigment. The reduced antibiotic activity noted when tap water was used in the prepartion of the medium may have been due to the inorganic contaminants. Waksman *et al.* (162) reported that the addition of Zn to media depressed production of fumigacin (a mixture of gliotoxin and helvolic acid) and appeared to favor its rapid destruction.

Aspergillus glaucus. Copper has been found essential for growth and spore production (93).

Aspergillus niger. The interest in the effects of metallic ions on the growth of this organism has continued during the past decade.

Steinberg's reports have dealt with effects of certain ions on the growth at 35° C. of a certain strain of *A. niger* on a sucrose-salts medium. His previous studies (see 41) have indicated the essentiality of Fe, Cu, Zn, Mn, Mo and Ga for this strain grown under these conditions. Mo seems to be of importance in reduction of NO_3, NO_2 and hydroxylamic acid (139), while all of the group are essential for maximum growth on media containing either amino acids or inorganic nitrogen (142, 144). He reports (140) that the source of carbohydrate does not influence the trace element requirements, and sucrose, glucose, fructose, mannose and sorbose all served equally well, while Sc (in addition to the other six) increased growth on gylcerol. A later investigation (145) confirmed these observations and indicated that the trace element requirements for maximum growth on media containing monoses as source of carbohydrate were similar to those with sucrose. The above mentioned six elements were required in approximately equal degrees, whatever state of oxidation of sulfur was supplied as nutrient (143). and omission of these six when CO_2 was absent from the atmosphere above the mycelium resulted in a greater reduction in growth than when CO_2 was present (146). In studies with a non-optimal medium increased growth was obtained when Ti, Cb and Pd were added (148), and Na and Be increased growth when the medium was deficient in K and Mg (149).

Other investigators have used other strains of *A. niger* and other laboratory conditions and media, and it is not surprising that results are often somewhat contradictory. Bertrand has observed (11, 12, 13) that the addition of V to medium containing Fe, Zn, Cu, Mn and Mo increased growth markedly. When starch was the carbohydrate source another investigator found that Zn, Cu, Fe and Mn were necessary for optimum growth (40). Zn has been reported as necessary for the utilization of glucose (for growth) from Raulin's medium (63) and the formation of cellulose (64). Neilsen (103) has summarized his observations by suggesting that mixtures of Cd, Ba, Sr, Ca, Li, Zn, Be, Cu and Mn in small quantities act as a co-growth substance. Mo is apparently necessary for maximum growth on a medium containing arabogalactan (116). Many have noted that *A. niger* will grow under what normally would be considered extreme conditions; an

example is the report that a culture grew on media containing 0.3 M in Al (22).

In a recent review (147) Steinberg has concluded that the mineral requirements of *A. niger* agree with those of green plants, and thus the use of this organism in testing soil samples has been understandably widespread. Spore color in these tests is associated with the presence of available Cu (2), and while Zn is also essential for growth it cannot replace Cu (58). The presence of Mn in soil samples tested by this procedure resulted in increased growth, while Al and Ti when present often had a stimulatory effect (104). Sporulation was reduced when Fe, Mo or Mn was absent, but not when Cu, Zn or Ga was missing (148). The *A. niger* growth test has been suggested for the qualitative testing of blood for the presence of Fe and Zn (95), as well as the testing of soil for available Mg (136). Zn, Fe and Cu are not necessary for spore germination (65).

Riboflavin production by *A. niger* mentioned in the previous review has been investigated further. While stimulation has been reported upon the addition of Hg to the medium (69), this has not been confirmed (27), and another report indicates that addition of $HgCl_2$ results in release of riboflavin into the medium (70). Traces of minerals present in tap water were unfavorable to production of flavin type pigments (73).

It is usually assumed that metallic ions exert their effects on specific enzyme systems, although concrete evidence on the mode of action is often lacking. Hydrolysis of arabogalactan was increased when Cu, Fe, Zn, Mn and Mo were added to the medium (116), and when the first four of this group were added to a starch medium, growth was increased, but amylase and starch decomposition were reduced (40). Zinc has been reported to be necessary for the utilization of glucose in Raulin's medium (63). Addition of Ra to a medium resulted in affected growth and subsequent production of amylase and proteinase (96, 97, 98). It seems likely that the effects of certain organic substances may be traced to their metallic ion impurities (141) and not to specific effects on enzyme systems.

Several organic acids of economic importance are produced by this organism, and it is not surprisng to find that metallic ions

affect the production of these chemicals. Several reports have indicated effects on total acid production only and have not mentioned specific acids. These include mention that addition of iron to Raulin's medium results in initial acid production and subsequent utilization of this acid (54) probably similar to observations on citric acid production (111). Another report indicated that added Zn and Fe had no effect on acid production from raw cane sugar (174).

Gluconic acid production was thought to be reduced by iron dissolved from the fermentation vessel (114), and in this connection the inhibitory effect of various metals often used for construction of fermentation vessels on citric acid production is of interest (135). In the latter report stainless steel strips placed in fermentation flasks resulted in reduced yields, while aluminum strips did not. It has been reported that the addition of Hg to a glucose or sucrose medium shifted the fermentation from a citric-oxalic type to gluconic acid (69).

Citric acid production in both surface and submerged culture has been investigated in a number of laboratories. Impurities in ash of beet sugar (9) and molasses (68, 105, 108) markedly affected production. Ferrocyanide treatment of molasses removed some of the inhibiting materials (14, 15, 49, 68, 108, 135).

When synthetic media have been used in surface culture, yields of citric acid and effects of metallic ions on citric acid production have varied with the strain of *A. niger* used. Bernhauer *et al.* (10) report that Mn had no consistent effect on citric acid production, and Sn, Al, Co, Ni, Cr, Se, Pd, Pt, Cu and Fe (all at 0.01%) had no effect, while Zn, Ag, Te, U, Sb and W inhibited citric acid production by a certain strain of *A. niger*. Other strains have been stimulated by the addition of Fe, Mn, Cr and Al (105, 109), although mixtures of these elements were not better than the single elements, and the optimum concentration varied from one strain to another. This latter qualification seems general, for the stimulatory effect of Se is reported to vary with the culture as well as the purity of the sugar (74). As might be expected from the foregoing, some cultures are more affected by impurities in the medium (usually from the water source) than others (75).

When a medium in which a treated beet molasses was used as source of fermentable carbohydrate, citric acid production (in surface culture) was reduced by the addition of salts of a number of elements, including Fe, Zn, Pb, Mn and Cu (68), as well as ash from certain untreated molasses (108).

In submerged culture the addition of Mn markedly reduced citric acid production by one strain of *A. niger*, although it had no effect on citric acid production in surface culture by this same strain (132, 133) and little effect in submerged culture by other strains (111). Fe was definitely necessary for maximum yields of acid (111, 132) when grown on a synthetic medium. Some strains require less Fe than others, and when high concentrations are added, yields are markedly reduced by these strains. On the other hand, other strains are relatively tolerant of high Fe levels (111). Mn, Zn, Al, Cr and other ions also affect production (60, 111). It has been shown that addition of Mn to the sporulation medium reduces the yield of citric acid produced when these spores are used as inoculum in fermenters (133), and that Fe and Cu did not exert this effect. Shu and Johnson (133a, 133b) have studied the effects of Fe, Zn, $MgSO_4$ and KH_2PO_4 on the production of citric acid and conclude that these factors are interdependent.

Aspergillus oryzae. Yoshimura (173) has studied the effect of metallic ions on the production of catalase by this organism. Addition of Fe, Zn, Cu or Mn at low concentrations to the medium favored catalase production, while higher concentrations of the latter two inhibited spherical cell formation and accelerated conidial production which generally counteracted catalase activity.

Aspergillus terreus. Production of itaconic acid by strains of this organism has been investigated in surface and submerged culture. Lockwood and Reeves (84) report that the addition of Mn, Mo, Co, Cu, Ni, Cr and Ga to the media containing salts and technical grade glucose had no effect on acid production. The inhibitory effect of Al was counteracted by the addition of $MgSO_4$. The stimulation of acid production on the addition of Fe and Zn depended on the pH of the medium. Moyer and Coghill (90) used another strain of this organism and found that the addition of Fe and Zn to a purified medium containing salts and glucose resulted

in increased acid production. Better results were obtained when the medium contained technical glucose than with CP glucose or sucrose. Mixtures of Cb and Zn, or Mo and Zn, were apparently equivalent to Fe and Zn mixtures in increasing acid production, and these investigators concluded that "with the possible exception of Zn there appears to be little gain from the use of trace elements."

In submerged culture the addition of Zn to a salts-glucose-corn-steep water medium resulted in no appreciable change in acid production (85).

Aspergillus wentii. Karow and Waksman (60, 61, 62, 163) have studied the production of citric acid in submerged culture by this organism, and conclude "the concentration of nutrients must be restricted to a minimum; barely sufficient to allow good mycelial development and not so high as to permit maximum growth of the organism." Highest yields of citric acid were obtained when small amounts of Mn and Zn were added to the medium. Addition of higher concentrations of Zn or Fe did not favor acid production, and certain levels of Mn favored pigment production and mycelial growth.

Aspergillus sp. Timmonin and Rouatt (158) and Wyllie (172) have studied the production of citrinin by a culture of *Aspergillus* of the *glaucus* group when grown on media containing glucose, fructose or sucrose and inorganic salts. Production was increased when Fe and Zn were added to the medium. Maple sugar was a better carbohydrate source than sucrose, perhaps because of its metallic ion content.

Cephalosporium sp. Starkey (137) isolated a culture of this organism and one of *Acontium velatum* growing in saturated $CuSO_4$ solution at pH 0.2.

Epidermophyton floccosum. The inhibitory effect of Zn on oxygen uptake (101) could be reversed by addition of Ca, $MgSO_4$ or $Na_2S_2O_3$ (102).

Fusarium graminearum. The production of an orange-yellow pigment on Czapek-Dox medium was increased by addition of Mn and Zn to the medium (94).

Fusarium hyperoxysporum. Texera (154) reports that addition of Zn to Czapek-Dox medium is necessary if two antibiotics are to be produced by this organism. If Zn is absent the glucose fermentation is incomplete and only the antibiotic present in the

mycelium is produced. Mn and Cu had no effect on the elaboration of these substances.

Fusarium oxysporum. Zentmeyer (175) reported that Zn reversed the fungistatic action of 8-hydroxyquinoline towards this organism, and suggested that Zn is the most important trace element for its growth.

Memnoniella echinata. Addition of Fe, Mn or Zn to a salts-glucose medium resulted in increased mycelial development, while traces of Ni, Co, Cr or Al decreased growth (110). The addition of histidine reversed the inhibitory effect of Co (110), as suggested by the previous studies by Burk *et al.* (26), on the combinations of histidine and Co.

Monosporium sp. Bojanovsky (21) found that organisms isolated from soils grew best on media containing added Fe.

Mucor pusillus. The growth-inhibiting effects of high levels of $MgSO_4$ can be reversed by the addition of $HgCl_2$ (120).

Mucor ramannianus. A symbiotic relationship between this organism and *Rhodotorula rubra* was found in media containing aspargine for thiamine. When other nitrogen sources were used Li, Co, Mo, Hg, U and Al were necessary additions to the medium (159).

Neurospora crassa and *N. sitophila.* The addition of a trace element mixture containing Zn, B, Mo, Mn, Fe and Cu to a number of nutrient solutions all containing Fe increased the growth rate slightly; the tartrate used in this medium was found to be contaminated with trace elements (125). In liquid culture traces of Zn have been found to be stimulatory to growth (23, 28, 152) and to repress sporulation (151).

Penicillium brevicaule. Bird and Challenger (16, 17) have continued their studies on methylation with this organism and report that addition of K_2TeO_3 to Czapek-Dox medium results in the formation of $(CH_3)_2Te$. The addition of sodium salts of R SeO_2H (R: CH_3, CH_3CH_2, or $CH_3CH_2CH_2$) resulted in formation of the mixed and di selenides of these precursors.

Penicillium chrysogenum. Recent interest in the effect of metallic ions on the growth and metabolism of this organism stems mainly from its role in the production of penicillin. It is probable that many of the effects noted will be observed in the closely related *P. notatum.* Jarvis and Johnson (55, 56, 57) have reported that

Zn, Fe and Mn were essential for optimum growth and penicillin production on a synthetic medium. Mn was necessary when a purified medium was used, as was Ca, but these two were not essential when a less pure media (U.S.P. ingredients) were used. They found no requirement for Cr, which had been reported (115) as stimulatory to penicillin yields. Pratt and Dufrenoy (115) could not replace Al and Cr by Mo, Co or Ni. Knight (71) and Koffler *et al.* (78) have tested a large number of the constituents of cornsteep liquor ash which had been found to stimulate penicillin production (72, 77), including Al, As, Cr, Co, Cu, Fe, Pb, Mn, Ni, Ag, Sn, W and Zn. The combination of Fe and phosphate was as effective as cornsteep ash in promoting penicillin production. Fe and Cr combinations also increased penicillin production, while Al antagonized the stimulatory effect of Fe. The presence of more than 2 ppm of Cu prevented accumulation of penicillin, but this effect was nullified by the addition of Fe. Woodruff (168) has commented that the heavy metal concentration of the medium had little effect on the differential bioassay (*Staphylococcus aureus—Bacillus subtilis* comparative response), although the final antibiotic potency of the broth was altered.

Other observations with this organism include that of Moyer (91) that the yield of gluconic acid was reduced when Zn was added to a glucose-salts-cornsteep liquor medium, and that of Bird and Challenger (16) that $(CH_3)_2Te$ and $(CH_3)_2Se$ were evolved when K_2TeO_3 or Na_2SeO_3, respectively, were added to Czapek-Dox medium or bread cultures.

Penicillium citrinum. Bailey and Cavallito (7) noted a 30-fold increase in the yield of citrinin when Fe was added to salts-glucose medium. Other studies (82) have shown that the Fe may be replaced by Mn but not by Cu or Zn.

Penicillium notatum. The recent studies on penicillin production by this organism have included several observations on the role of metallic ions in the biosynthesis of this substance. It is probable that many of the reports dealing with this organism apply equally to the closely related *P. chrysogenum.* In their early paper the Oxford group (1) noted that the use of tap water instead of distilled water did not result in any change in penicillin yield. Other studies with this organism in surface culture (30) seemed to indicate that the addition of Cu increased penicillin yield; while this

effect was difficult to repeat, it was noted that pigment production was lower in media containing additional Cu. Other studies (surface culture) by Cook *et al.* (31, 32, 33) indicated that the addition of pea extract increased penicillin production, and that part of the stimulation could be traced to the presence of metallic ions in the extract ash. Foster *et al.* (45) noted that the addition of Zn to a glycerol-salts medium increased penicillin production in the presence or absence of Fe, Mn or Cu. He also noted that some of the superiority of brown sugar media over those containing purified sugars could be found in the ash of the brown sugar. Moyer and Coghill (91) report that *P. notatum* showed a typical trace element requirement in a purified synthetic medium. They added a large number of elements, including Zn, Fe, Cu, Mo, Mn and Cb, to a synthetic medium, and found that only the addition of Zn of this group resulted in increased penicillin yield and also growth. The production of gluconic acid during the penicillin production phase on this medium was reduced when Zn was added to the medium. On the other hand, Negroni (99), studying this problem with another culture, has reported that the addition of Zn to a synthetic medium containing starch had no effect on penicillin production in surface culture.

When submerged culture methods were used, somewhat different results were observed. However, as a different strain was usually used in submerged culture, some of these observations may be the result of strain differences. Foster *et al.* (46) tested the effect of the addition of a number of elements to a brown sugar-salts medium on penicillin production and found that better yields of penicillin were obtained when Zn was added to the medium. He observed no apparent effect when Ca, Fe, Mn, Cu, Cd, Mo or W was added. Moyer and Coghill (92) found that the addition of Mn or Zn to a medium containing cornsteep liquor had no effect on penicillin production. Perlman (107) added Fe, Cr, Mn, Al, Sn and Cu to a synthetic medium and noted that when any one of the first five was added yields of penicillin were reduced. Stefaniak *et al.* (138) report that metal toxicity experiments indicated that aluminum and allegheny metal were nontoxic, while iron exhibited slight toxicity. A total iron content of 500 ppm or more reduced yields of penicillin in their laboratory. Gilbert and Hickey (50) have stated that the addition of appreciable quantities of iron to a cornsteep medium

caused decrease in penicillin production and increase in conidia formation. Foster *et al.* (43) mention that heavy metals affect conidiospore formation in submerged cultures by this organism.

Notatin (glucose aerodehydrogenase) production by this organism was reduced when Zn was added to the medium (35), and addition of Cu and Fe was necessary for maximum production of this enzyme. Impurities in brown sugar were responsible for increased production of notatin, according to other investigators (122).

Bird and Challenger *et al.* (16) have noted that this organism produces $(CH_3)_2Te$ and $(CH_3)_2Se$ when the respective salts were added to media, as had been observed with *P. chrysogenum.*

Penicillium patulum. Birkinshaw *et al.* (18, 19) have reported that this organism will produce patulin and gentisyl alcohol when grown on Raulin-Thom medium. Brack (24) has studied this further and observed that increasing the quantity of iron in the medium resulted in increased production of gentisyl alcohol and reduced production of patulin. Cu in presence of Fe reduced production of both substances and increased sporulation, while Zn in presence of Fe increased gentisyl alcohol and reduced sporulation. The addition of Mn to the medium containing Fe increased sporulation, eliminated production of gentisyl alcohol and increased patulin yield.

Penicillium phoeniceum. Curtin *et al.* (36) observed that the addition of Zn to a synthetic medium reduced pigment production.

Penicillium terlikowskii. The increase in gliotoxin production on media containing impure sugar, as compared with media containing purified sugar, was attributed to impurities, probably inorganic in nature (25).

Penicillium waksmanii. This organism is reported (51) to be able to grow in a saturated solution of copper sulfate as well as in concentrated solutions of $ZnSO_4$ and $MnCl_2$. Sporulation and spore germination are retarded by even dilute solutions of $CuSO_4$.

Penicillium sp. An apparent association was observed between mold growth and treatment of cut potato tubers with salts of Cu, Co, Ni and Fe (38). Good growth was obtained on tubers moistened with 2.5% Cu and Co salts; less stimulation was noticed when Ni and Fe were used, and only slight stimulation occurred when Hg was used (37).

Zentmeyer (175) noted that when 8-hydroxyquinoline was added to a medium whose pH was above 3.2, growth of a *Penicillium* culture was inhibited, and this effect could be reversed by the addition of Zn.

Phycomyces blakesleeanus. Leonian and Lilly (79) found that the addition of agar ash or Zn to a purified medium containing thiamin, salts, amino acids and glucose doubled growth. The addition of Cu, Ga, Mn, Mo and Fe singly or in combination had no effect. When the medium was purified by the $CaCO_3$ adsorption procedure, a stimulation of growth was noted when Fe was added to the medium. A later report (80) indicated that the addition of Mo, Mn or Ga to a medium containing Fe and Zn had no effect on zygospore formation. Production of biotin by this organism on a similar medium was increased by the addition of a mixture of salts containing Cu, Fe, Mn, Zn, Ga and Mo (127).

Utiger (160) suggests that the metallic ions present in plant material interfere with the bioassay for thiamin, utilizing this organism, as indicated by the discrepancy between the values obtained with the thiachrome method and the *Phycomyces* growth test. Schopfer (129) does not think that Utiger's conclusions were justified.

Phymatotrichum omnivorum. Experiments by Blank (19*a*), studying the effect of the addition of single elements to a basal medium, indicated that the addition of Zn, Mn or Fe to an inorganic salts-glucose medium (purified by the $CaCO_3$ absorption technique) increased mycelium formed, while addition of Al, Cd, Co, Ni or Cu resulted in reduced mycelium growth, and addition of Si or Mo under the same conditions had no effect. He also observed that the addition of Al, Cd, Co, Hg, Mo, Ni or Si to the basal medium containing Mn, Fe and Zn did not result in increased mycelium growth, and concluded that these elements are not essential for optimum growth. These observations had been previously summarized by Rogers (123*a*). Ezekiel's studies (39*a*) on a similar medium indicated that a mixture of Zn, Cu, Mn and Fe increased mycelium growth when added to the basal medium.

Polyporus adustus and *P. abietinus.* Fries (47) did not find that the addition of Zn, Cu and Mn to a glucose-salts (including Fe) medium affected growth.

Polyporus vaporarius. This organism produced enough oxalic

acid to reverse the growth-inhibiting effect of $CuSO_4$ present in impregnated timbers (67).

Rhizopus delemar. Erb and Hildebrandt (39) found that the addition of aluminum powder to a mash containing stillage permitted heavy growth. It was first thought that the aluminum counteracted some toxicity due to traces of other metals picked up during mashing or distilling, but this was not a complete explanation, since stillage produced in glass was similarly toxic for the mold until treated with aluminum.

Rhizopus oryzae. Lockwood (83) notes that addition of Zn to a glucose-salts medium resulted in more rapid germination of spores. In submerged culture the Zn did not influence acid production, while it reduced it in surface culture.

Rhizopus suinus. Nielsen (103) has observed that a mixture of metals, including Cd, Ba, Sr, Ca, Li, Be, Zn, Cu and Mn in small quantities, acted as a co-growth substance. Others have deduced that the stimulation of growth found by adding glucose ash (34) or filter paper ash (170, 171), also called cofactor B, was apparently an inorganic salt of metallic ions, perhaps Fe or Zn.

Rhizopus sp. Foster and Waksman (44, 161), as mentioned in the previous review (41), have noted that addition of Zn to the medium results in increased growth and reduced fumaric acid and ethanol accumulation. Zn apparently stimulates the growth and respiratory coefficient of the organism, but is not favorable to the accumulation of lactic acid.

Sclerotium delphinii. While addition of Fe, Zn, Cu, or Mn did not increase growth on a synthetic medium, the addition of Zn or Mn increased color of the sclerotium. Addition of Cr, Al, Co, or Ni resulted in significant inhibition of growth, while Fe resulted in increased rate of oxalic acid decomposition (111a).

Streptomyces griseus. In surface culture Mn can be substituted for Fe on a medium containing salts-beef extract and glucose without affecting streptomycin production (3). Addition of Zn gave rise to rapid pellicle formation but inhibited streptomycin production, while Fe had a reverse effect (167). Traces of Zn, Cu, Fe and Mn were found necessary by Thornberry (156) for optimum production of streptomycin in a synthetic medium.

Saunders and Sylvester (126) report that traces of Cu, Mn, Fe and Zn were necessary ingredients of synthetic media to obtain

maximum streptomycin yields when the organism was grown in submerged culture. Bennett (8) observed that much of the stimulation of streptomycin production due to cornsteep liquor could be obtained by inclusion in the medium of a proper concentration of cornsteep liquor ash, or potassium salts, such as potassium phosphate or chloride. Thornberry's recent note (157) confirms this, in that he found that ash from a number of natural materials, including peanut meal, sunflower meal, distiller's solubles and cornsteep water, increased streptomycin production when added to a medium containing glucose, peptone and NaCl.

Streptomyces scabies. One race of six tested grew on media containing 100 ppm of $HgCl_2$ (155).

Streptomyces sp. A culture resembling *S. erythreus* and *S. albosporus* required Fe for the production of actinorubin. The presence of excess Fe resulted in acid production (66).

Trichophyton rubrum and *T. gypseum.* Nickerson (101, 102) has observed that the addition of $ZnCl_2$, $Zn(NO_3)_2$, $CdSO_4$, $AgNO_3$ or $HgCl_2$ to the medium reduced the oxygen uptake of cell suspension of these organisms. The inhibition effect caused by the Zn salts could not be reversed by the addition of $CaCl_2$, $Na_2S_2O_3$ or $MgSO_4$. Addition of $CdCl_2$ increased the oxygen uptake.

DISCUSSION

While a number of advances have been made in the recognition of the effects of metallic ions on the physiology of fungi, as evidenced by the scope of the investigations mentioned, there are a number of rather basic questions still to be studied in detail. There have been numerous papers indicating that a certain effect is traceable to the presence of certain unidentified inorganic constituents (ash of a natural material—usually one of the medium constituents) (8, 9, 25, 31, 32, 33, 34, 46, 58, 68, 71, 72, 75, 108, 121, 135, 157, 159, *etc.*). Whether these effects can be attributed to a certain ion or a mixture of ions, or whether these effects are the results of imbalance of ions, or the "complexing" of an ion, is often not settled. Very often the effect may be reproduced by the addition of a single known ion or a mixture of ions, and this is taken to be proof that the effect of the ash (or similar material) was due to the presence of this ion mixture. From the data at hand it appears that the effects of one ion counteracting the effects of another

ion are often overlooked in this problem, and it seems probable that this phenomenon should be considered in explaining some of the effects of the additions of such materials as ash or residues to certain media.

Thus far the problem of essentiality of any given ion for growth or a certain metabolic activity has only been touched upon. Apparently no reports are available indicating that a certain ion is required to obtain a minimum growth, a condition encountered in the study of the growth-factor requirements of fungi where specificity is very marked (128). Leonian and Lilly (81) state "The food constituents which are least affected by the specific requirements of these test organisms are the mineral elements", and this has apparently been the feeling of many other investigators who have routinely added a standard mixture of ions to various media when studying the growth-factor requirements of various organisms, or the effects of various chemical substances or physical factors on the growth or metabolism of fungi. This may often lead to erroneous conclusions, since many of these organic substances combine with metallic ions and thus affect the physiology of the test organism by making these substances unavailable to the organism. It has also been shown many times (46, 48, 71, 79, 109, 110, 145) that the addition of any one of a number of elements will result in increased growth or metabolic activity on a medium which supports only minimal growth or metabolic activity. The substitution of one element for another is especially noticeable if growth is taken at the end-point, although, as Foster (42) points out, this substitution may result in a varying composition of cellular material as well as a shunting of the carbohydrate metabolism of the mold. Perhaps this also occurs when analogues of various growth-factors are substituted; apparently, no observations have been made. The effect of these substitutions is also puzzling, for approximately the same results are apparently obtained with several substitutions (90, 106). Does this indicate that the enzymatic systems involved may be catalyzed by several different ions, or that several alternative mechanisms exist, or is this confirmation of Hutner's (53) suggestion that the contaminants in the added ions are responsible for the apparent effects. It might be noted here that very often poisonous substances, such as those containing mer-

cury (119, 123, 134), act as growth promoters, but perhaps this is a manifestation of the Shultz-Arnst effect.

Other interesting developments are the reports concerning the effects of metallic ions on spore formation, and subsequent fermentations by these spores (111, 132). An explanation for this phenomenon is that certain metallic ions favor the formation of a certain type of spore, or perhaps the composition of the spore varies with the metallic ion content of the sporulation medium. This may account in part for the apparent variation in cultures observed by Chrzaszcz and Zakomorny (29). This observation is in line with the effect of metallic ions on the composition of the cellular material (112, 131).

A consideration of the literature cited in this review suggests that several rather basic criticisms may be applied to many of the investigations reported, and in turn these criticisms limit any generalizations that may be drawn from the observations at hand. Since many of the reports deal with *A. niger,* the work on this organism may be used as an illustration. The reports indicate that a large number of different cultures have been used which in turn have been obtained from various sources and consequently have different histories. Usually these cultures have been grown on different media and under different cultural conditions in each laboratory. Very rarely is the trace element content of the medium stated or mentioned, and a comparison (111) has indicated that different strains of the organism react differently under the same cultural conditions when grown on the same medium. It has been reported that such matters as temperature of incubation will cause radical changes in growth (13) as well as metabolism of various strains (111). Until some effort is made to standardize techniques, as growing inoculum, sporulation, methods of preparing stock cultures, preparation of media, *etc.,* it is inadvisable to draw any general conclusions regarding the metabolism of carbohydrates by fungi, or the effects of metallic ions on the metabolism, as indicated by the few comparisons that have been made between cultures grown under different laboratory conditions (13, 107, 111, 132).

The above discussion has mentioned only a few of the prominent problems that exist on this general topic. During the nine years covered by this review, a number of subjects have generally been

recognized in the various laboratories studying physiology of fungi, including metallic ion antagonism, lack of specificity of metallic ions for certain responses, and the indirect effects of metallic ions on the fermentation by their effects on sporulation. However, these topics are not new and have been mentioned in the literature many years ago. Perhaps the investigations of the next decade will resolve some of these old problems as well as open others.

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